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Metagenomic cloning and characterization of Na^+ transporters from Huamachi Salt Lake in China

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ABSTRACT

Moderately halophilic bacteria are a kind of extreme environment microorganism that can tolerate moderate salt concentrations ranging from 0.5 M to 2.5 M. Here, via a metagenomic library screen, we identified four putative Na^+ transporters, designated H7-Nha, H16-Mppe, H19-Cap and H35-Mrp, from moderately halophilic community in the hypersaline soil of Huamachi Salt Lake, China. Functional complementation observed in a $Na^+(Ca^{2+})/H^+$ antiporter-defective *Escherichia coli* mutant (KNabc) suggests that the four putative Na^+ transporters could confer cells a capacity of Na^+ resistance probably by enhancing Na^+ or Ca^{2+} efflux, but not Li^+ or K^+ exchange. Blastp analysis of the deduced amino-acid sequences indicates that H7-Nha has 71% identity to the NhaG Na^+/H^+ antiporter of *Bacillus subtilis*, while H19-Cap shows 99% identity to *Enterobacter cloacae* Ca^{2+} antiporter. Interestingly, H16-Mppe shares 59% identity to the metallophosphoesterase of *Bacillus cellulosilyticus* and H35-Mrp shows 68% identity to multidrug resistance protein of *Lysinibacillus sphaericus*. This is the first report that predicts a potential role of metallophosphoesterase in Na^+ resistance in halophilic bacteria. Furthermore, everted membrane vesicles prepared from *E. coli* cells harboring H7-Nha exhibit Na^+/H^+ antiporter activity, but not Li^+ $(K^+)/H^+$ antiporter activity, confirming that H7-Nha supports Na^+ resistance mainly via Na^+/H^+ antiport. Our report also demonstrates that metagenomic library screen is a convenient and effective way to explore more novel types of Na^+ transporters.

1. Introduction

Moderately halophilic bacteria are a class of eubacteria that grow optimally in the environments with salt concentration between 0.5 M and 2.5 M (Kushner 1985; Monteoliva-Sanchez et al. 1993). These bacteria are widely distributed in salt lakes, saline land, coastal lagoons, deserts, ocean and so on (Rodriguez-Valera 1986; Simon et al. 1994; Grant 1996; Liu et al. 2005a,b). To adapt to these environments, most of them have developed a series of sodium transport systems, which may play vital roles in the Na^+ cycle that supports pH homeostasis, salt resistance, solute uptake, and motility (Padan et al. 2001, 2004; Liu et al. 2005a,b). Today, more and more halophilic species and genera are proposed in the family Halobacteriaceae, while the mechanisms of their sodium transport system or functions of related genes remain to be well understood.

In general, based on source of energy, sodium transport systems can be divided into two groups: primary sodium pump and secondary transporters. Primary sodium pump directly uses chemical energy, such as adenosine triphosphate (ATP), redox energy or photon energy (light), to transport molecules across a membrane (Yamakawa et al. 1989; Häse et al. 2001). A large number of enzymes have been proposed to perform this type of transport, e.g., decarboxylases, coenzyme M methyltransferases, ATP synthases, and NADH-ubiquinone oxidoreductases (Yamakawa et al. 1989; Kaim 2001; Studer et al. 2007). Secondary Na^+/H^+ antiporters are a subset of intergral membrane proteins, which play an essential role in transport molecules or ions across the membrane. In contrast to primary sodium pump, secondary antiporters use its own electrochemical gradient as a source of energy. The family of secondary Na^+/H^+ antiporters as an example, contains a large number of bacterial Na^+/H^+ antiporter proteins (Horn 2000; Padan 2008). In recent years, studies are mainly focus on the identification and characterization of *Escherichia coli* Na^+/H^+ antiporter proteins, including single-subunit antiporter proteins, e.g., NhaA (Padan et al. 2009), NhaB (Herz et al. 2003), NhaD (Kurz et al. 2006), NhaG (Gouda et al. 2001), NhaP (Waditee et al. 2006). Besides, similar research has been carried out in *Bacillus subtilis*. Several cation/H^+ antiporters, such as Mrp (Ito et al. 1999), NhaK (Fujisawa et al. 2005), Tet (L) (Cheng et al. 1996), NhaG (Gouda et al. 2001) and NhaC (Pragai et al. 2001) have been proposed to function in sodium transport system of *B. subtilis*.

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In this study, we isolated and characterized four putative antiporters by screening a metagenomic library which was generated with genomic DNA extracted from the hypersaline soil samples of Huamachi Salt Lake located in Northwest of China. Sequence analysis demonstrated that these four putative antiporters are new members of sodium transport system that regulates sodium homeostasis in halophilic bacteria. In addition, functional complementation assay revealed that the Na^+ (Ca^{2+})-sensitive phenotypes of *E. coli* KNabc (Δ*nhaA* Δ*nhaB* Δ*chaA*) could be restored by introducing H7-Nha into the cells, which further confirmed the Na^+/H^+ transport activity of H7-Nha protein.

2. Experimental procedures

2.1. Strains and growth conditions

E. coli strain KNabc (Δ*nhaA* Δ*nhaB* Δ*chaA*) (Nozaki et al. 1996), which is unable to grow in the medium with 0.2 M NaCl, was routinely cultivated in the LBK medium containing 10 g/L tryptone, 5 g/L yeast extract, and 6.5 g/L potassium chloride at pH 7.0. *E. coli* strain JM109 used as the host of metagenome clone was cultivated at 37 °C in Luria-Bertani medium containing 10 g/L tryptone, 5 g/L yeast extract, and 10 g/L sodium chloride at pH 7.0. If needed, antibiotics were added at an initial concentration of 100 mg/L of ampicillin or 50 mg/L of kanamycin in the medium.

2.2. Construction of metagenomic library from Huamachi Salt Lake in China

Soil samples from five spots in Huamachi Salt Lake of China were obtained for metagenomic library construction. Each sample was inoculated into a 250 mL flask with 100 mL of 8% Gibbson medium (10 g tryptone, 5 g yeast extract, 5 g casein, 2 g KCl, 3 g sodium citrate, 20 g $MgSO_4 \cdot 7H_2O$, 80 g NaCl in each liter) and incubated at 30 °C for 2–3 days. Then 1 mL of the first cultures was transferred to the same medium for one more time. After 16 h of incubation at 30 °C with shaking, all the cells were harvested by centrifugation, and DNA preparation were carried out as described by Yoon et al. (1996) with a slight modification. The DNA mixture was partially digested with *Sau*3A I and fragments ranging from 4 kb to 6 kb were recovered from the gel and cloned into the *Bam*H I site of pUC118 cloning vector. The ligation product was introduced into *E. coli* strain JM109, and transformants were selected on LB plates with 100 mg/L ampicillin (pH 7.0). The plasmid DNA extracted from these transformants were collected and introduced into *E. coli* strain KNabc to construct metagenomic library.

2.3. Screening and cloning of Na^+/H^+ antiporters from the metagenomic library

The metagenomic library was screened via complementation experiment by checking the growth characteristics of *E. coli* strain KNabc (Δ*nhaA* Δ*nhaB* Δ*chaA*) in LBK medium with 0.2 M NaCl. Clones that can grow in the medium mentioned above were selected and the DNA fragments inserted in the plasmids were sequenced further. The complete putative Na^+/H^+ antiporter genes including the promoter (300 bp upstream of the ATG) and terminator region (200 bp downstream of the stop codon) were subcloned into plasmid pUC118, yielding recombinant plasmids pUCH7-nha, pUCH16-mppe, pUCH19-cap and pUCH35-mrp.

2.4. Growth experiments

The growth of *E. coli* strain KNabc carrying the blank vector pUC118 or Na^+/H^+ antiporter genes containing plasmid was determined in LBK medium supplemented with different Na^+ concentration (0–0.6 M). Cell density was monitored at 600 nm. The results are representative of triplicates of individual cultures.

2.5. Preparation and assays of everted membrane vesicles

E. coli strain KNabc carrying the blank vector pUC118 or Na^+/H^+ antiporter genes were pregrown in LBK medium at pH 7.0, and everted membrane vesicles were prepared as described by Rosen (1986). The Na^+/H^+ antiport activity was determinated by the quinacrine fluorescence quenching method (Goldberg et al. 1987). ΔpH in everted membrane vesicles (80 μg of protein) was monitored with acridine orange (1 μM) in a buffer containing 10 mM Tris-MES (pH 8.0), 140 mM choline chloride and 5 mM $MgCl_2$ at pH 8.0. At the onset of the experiment, potassium L-lactate (5 mM) was added and the fluorescence quenching (Q) was recorded by Hitachi F-4500 fluorescence spectrophotometer. NaCl, KCl or LiCl (10 mM) was then added, and the new steady state of fluorescence was obtained (dequenching) and each addition was monitored.

3. Results

3.1. Construction and analysis of metagenomic library from Huamachi Salt Lake

As described in Section 2, a metagenomic library was constructed by using genomic DNA obtained from the hypersaline soil samples of Huamachi Lake and with pUC118 as a vector. Based on the ability of the library-containing Na^+ (Ca^{2+})/H^+ antiporter defective mutant *E. coli* KNabc (Δ*nhaA* Δ*nhaB* Δ*chaA*) when grown on medium containing 0.2 M NaCl, a total of 41 clones conferring salt transport activity were isolated from 52,100 clones. After subcloning and sequence analysis, 4 different genes, termed *H7-Nha* (H7 *Na*$^+$/*H*$^+$ *a*ntiporter), *H16-Mppe* (H16 metallo*p*hos*p*hoesterase), *H19-Cap* (H19 *C*a^{2+} *a*nti*p*orter) and *H35-Mrp* (H35 *m*ultidrug *r*esistance *p*rotein), were obtained. Sequence comparison data indicate that the *H7-nha* open reading frame (ORF) is composed of 1578 bp and is predicted to encode a 525 amino acid protein (57.9 kDa), the *H16-mppe* ORF comprises of 838 bp and encodes a 274 aa-length protein (30.9 kDa). It is also demonstrated that the *H19-Cap* ORF is 1101 bp and encodes a protein of 366 amino acids with the molecular weight 39 kDa, the *H35-Mrp* ORF comprises of 342 bp and encodes a 113 aa-length protein. Subsequent amino acid sequence alignment indicates that H7-Nha and H19-Cap show high identity to Na^+/H^+ antiporter NhaG of *Bacillus subtilis* (71%) and *Enterobacter cancerogenus* Ca^{2+} antiporter (99%), respectively, suggesting that H7-Nha and H19-Cap are two new members of secondary antiporter family, in which H7-Nha is grouped to NhaG-type Na^+/H^+ antiporters, H19-Cap belongs to divalent cation/H^+ antiporters (Fig. 1a and c). In addition, H16-Mppe was found to share 59% identity to metallophosphoesterase of *Bacillus cellulosilyticus* and H35-Mrp show 68% identity to multidrug resistance protein of *Lysinibacillus sphaericus*, implying a strong probability that these two proteins possess the primary energization capacity (Fig. 1b and d). Moreover, hydrophobicity analysis showed that all the four putative transporters except H16-Mppe contain an excess of acidic amino acids over hydrophilic residues, implying that H7-Nha, H19-Cap and H35-Mrp, but not H16-Mppe are membrane proteins (Fig. 1).

3.2. Growth of E. coli KNabc carrying H7-Nha, H16-Mppe, H19-Cap or H35-Mrp in different concentrations of Na^+

In order to further test roles of the four putative transporters in salt tolerance, the *E. coli* KNabc strains carrying *H7-Nha*, *H16-Mppe*, *H19-Cap* or *H35-Mrp*, were inoculated into liquid media containing